

## Identification of T-cell receptor $\alpha$ -chain genes in the chicken

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**ABSTRACT** T-cell receptor (TCR)  $\alpha$ -chain (TCR $\alpha$ ) and  $\beta$ -chain (TCR $\beta$ ) genes are well characterized in mammals, while only TCR $\beta$  genes have been identified in other vertebrates. To identify avian TCR $\alpha$  genes, we used monoclonal anti-CD3 antibodies to isolate chicken TCR $\alpha$  for peptide sequence analysis. Degenerate oligonucleotide probes were then used to isolate a candidate TCR $\alpha$  cDNA clone that hybridized with a 1.7-kb mRNA species present only in  $\alpha\beta$  T cells and in tissues populated by these cells. Southern blot analysis revealed gene rearrangement in thymocytes and  $\alpha\beta$  T-cell lines. The TCR $\alpha$  cDNA candidate encoded an open reading frame of 275 amino acids, the predicted variable (V)-, joining (J)-, and constant (C)-region amino acid sequences of which shared  $\approx 40\%$ ,  $60\%$ , and  $25\%$  homology with corresponding mammalian sequences. A single C $\alpha$  gene and  $\approx 25$  V $\alpha$  genes were identified by using region-specific probes. The V $\alpha$  cDNA probe isolated from a V $\beta 1^+$  cell line reacted with transcripts from one of five V $\beta 2^+$  cell lines, suggesting shared use of V $\alpha$  genes by V $\beta 1^+$  and V $\beta 2^+$  T cells and the existence of other V $\alpha$  gene families. A genomic V $\alpha$  sequence was flanked by classical recombination signal sequences but, unlike previously defined V genes, the leader and V $\alpha$  region were encoded by a single exon. The data indicate evolutionary conservation of the basic TCR $\alpha$  gene structure in birds and mammals.

The T-cell systems of birds and mammals share many conserved structural and functional features (1). However, studies conducted in the chicken suggest a relatively simple genetic organization of avian  $\alpha\beta$  T-cell receptor (TCR) genes. The TCR  $\beta$ -chain (TCR $\beta$ ) gene locus in chickens contains two variable (V) region  $\beta$  families, V $\beta 1$  and V $\beta 2$ , each of which consists of very few highly homologous members (2). The avian V $\beta 1$  gene segments are rearranged by a deletional mechanism, whereas inversion is apparently the mechanism used to rearrange V $\beta 2$  genes (3). Monoclonal antibodies (mAbs) reacting with the gene products of either the avian V $\beta 1$  or V $\beta 2$  family have been used to identify two distinct  $\alpha\beta$  T-cell subpopulations that differ in ontogeny, tissue distribution, and function (4–8). The V $\beta 1^+$  cells appear earlier than the V $\beta 2^+$  cells during thymic development and begin migration out of the thymus to reach the peripheral lymphoid tissues by embryonic day 17, whereas the V $\beta 2^+$  cells begin to leave the thymus after hatching 3 or 4 days later. Both subpopulations home to the periarteriolar areas of the spleen, whereas most of the intestinal  $\alpha\beta$  T cells are V $\beta 1^+$  (7, 8), except in birds selectively depleted of V $\beta 1^+$  T cells when the V $\beta 2^+$  cells replace the missing V $\beta 1^+$  cells in the intestine. In these animals mucosal IgA antibody responses are severely compromised (9), suggesting that V $\beta 1^+$  T cells selectively promote the IgA isotype switch in mucosal antibody responses.

As a next step in analyzing the functional behavior of the prototypic V $\beta 1^+$  and V $\beta 2^+$  subpopulations of avian  $\alpha\beta$  T cells, we sought to identify the genes encoding TCR  $\alpha$  chains (TCR $\alpha$ ) in birds. Here we report the identification of a chicken TCR $\alpha$  gene family that includes multiple V $\alpha$  and J $\alpha$  genes and a single constant (C) region  $\alpha$  gene.††

### MATERIALS AND METHODS

**Cell Lines, mAbs, and DNA Libraries.** The Marek disease virus-induced V $\beta 1^+$  T-cell lines CU12, CU16, CU24, and CU36; the V $\beta 2^+$  T-cell lines CU15, CU38, CU41, CU132A, and CU147; the B-cell line CU68 (provided by K. A. Schat, Cornell University) (10); the Reticuloendotheliosis virus-T-induced B-cell line DT40 (E. Humphries, West Virginia University); the UG9 T-cell line (L. Schierman, University of Georgia); the SFP13 T-cell line (M. Linial, Fred Huchins Cancer Research Center, Seattle); the  $\gamma\delta$  T-cell line 857/2 (T. Graf, European Molecular Biology Laboratory, Heidelberg); and the macrophage cell line BM2 (R. Dietert, Cornell University) were maintained in a humidified atmosphere at 41°C and 5% CO<sub>2</sub>/95% air in RPMI 1640 medium/10% fetal calf serum/1 mM glutamine/penicillin at 100 units per ml/streptomycin at 100  $\mu$ g/ml.

The CT3 and CT4 mAbs recognizing the avian CD3 and CD4 homologues, respectively, were produced in our laboratory (11, 12). Two  $\lambda$ gt11 cDNA libraries were constructed from the UG9 cell line (V $\beta 1^+$ ) and the CU132A cell line (V $\beta 2^+$ ), as described (3). The  $\lambda$ gt10 chicken thymocyte library, as well as the erythrocyte DNA  $\lambda$ fix genomic library, was provided by C. Thompson (University of Chicago) (2). A liver DNA cosmid library was purchased from Clontech. Erythrocytes of different chicken strains were obtained from K. A. Schat (Cornell University).

**Protein Purification and Peptide Sequencing.** Plasma membranes of  $2.5 \times 10^{11}$  V $\beta 1^+$  UG9 cells were prepared according to standard procedures and solubilized in 10 mM Tris-HCl, pH 7.6/150 mM NaCl/0.3% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)/protease inhibitors. The solubilized membrane proteins were cleared by rotating with anti-CD4 mAb conjugated to Affi-Gel beads (Pierce) and then rotated with Affi-Gel bead-conjugated

Abbreviations: TCR, T-cell receptor; TCR $\alpha$  and TCR $\beta$ , TCR  $\alpha$  and  $\beta$  chains, respectively; V, variable; J, joining; C, constant; subscripted  $\alpha$  and  $\beta$  indicate  $\alpha$  and  $\beta$  chain, respectively; GPDH, glyceraldehyde-3-phosphate-dehydrogenase; mAb, monoclonal antibody.

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††The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U04611 and U04614).

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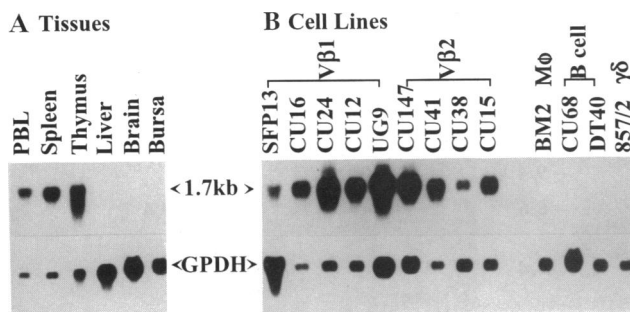


FIG. 1. RNA blot analysis of TCR $\alpha$  gene expression. Total RNA from different chicken tissues (A) and cell lines (B) was hybridized to the radiolabeled 1.7-kb candidate TCR $\alpha$  cDNA probe (Upper) and a probe specific for GPDH (Lower). PBL, peripheral blood lymphocytes.

anti-CD3 mAb. After being washed, the TCR-CD3 complex proteins were eluted with 0.1% trifluoroacetic acid and lyophilized. The proteins were then solubilized in H<sub>2</sub>O, reduced with 2-mercaptoethanol, and separated by SDS/PAGE (13). After transfer to a poly(vinylidene difluoride)-nitrocellulose membrane, the filters were stained with amido black 10 B (14), and the region of the membrane containing the 40-kDa protein was excised and digested with trypsin. The resulting peptides were eluted off the membrane and fractionated by reverse-phased HPLC. Pure peptides were analyzed on an automated gas/liquid-phase sequencer as described (15).

**Library Screening, DNA Sequencing, and Southern and RNA Blot Hybridization.** One 17-bp-long oligonucleotide pool (GAAR-AAAY-AAAR-CAY-GAR-GC) with 32 times degeneracy was synthesized on a Applied Biosystems model 380B DNA synthesizer and used to screen the thymocyte cDNA library according to the procedure of Wozney (16). The UG9 and CU132A cDNA libraries were screened with cDNA probes as described (17). Inserts of positive clones were subcloned into the Bluescript vector (Stratagene), and both strands of double-stranded DNA were sequenced with vector and sequence-specific primers by using a Sequenase kit (United States Biochemical). The DNASTAR software package was used for sequence data analysis.

Isolation of RNA and DNA, RNA, and Southern blot analysis were done as described (17). A truncated clone specific for the C $\alpha$  region but not containing any joining (J)  $\alpha$  or V $\alpha$  sequences was used as a C-region-specific probe. A 635-bp-long *EcoRI*-*Pvu* II fragment was prepared as V $\alpha$  probe. A 42-bp oligonucleotide spanning the amino-terminal part of the J region was used as J $\alpha$  probe. A cDNA probe specific for glyceraldehyde-3-phosphate-dehydrogenase (GPDH) was used as control of RNA integrity.

## RESULTS AND DISCUSSION

**Isolation of a TCR cDNA Clone.** The TCR $\alpha\beta$ -CD3 complex of membrane proteins isolated from the V $\beta$ 1<sup>+</sup> T-cell line UG9 by an anti-CD3 affinity column was disassociated by reduction, and the constituent chains were resolved by gel electrophoresis to allow isolation of the 40-kDa TCR $\alpha$ . Peptide fragments of the TCR $\alpha$  proteins purified by HPLC yielded nine different amino acid sequences, none of which shared obvious homology with mammalian TCR $\alpha$  when compared with the SwissProt protein data bank. A stretch of 5 amino acids in the longest of these peptides, which gave the least degeneracy of oligonucleotides, was chosen to design probes. When 17-bp-oligonucleotide pools with 32 times degeneracy were constructed and used to screen a thymocyte cDNA library, one specific clone with a 0.9-kb insert was isolated. This cDNA reacted with a 1.7-kb RNA species in  $\alpha\beta$  T-cell lines but did not react in a macrophage cell line by RNA blot analysis (data not shown). Because sequence analysis of this clone revealed similarity to the mammalian TCR  $\alpha$ -chain genes, it was used to screen a T-cell line library (UG9) in an attempt to obtain a full-length TCR $\alpha$  cDNA clone. Of the 20 clones identified, the clone with the longest insert size (1.7 kb) was selected for further analysis. Using this clone as a probe, a 1.7-kb mRNA species was detected in  $\alpha\beta$  T-cell lines, thymus, spleen, and peripheral blood lymphocytes but was not detected in a  $\gamma\delta$  T-cell line, a macrophage cell line, B-cell lines, or in brain, liver, and bursa (Fig. 1). This tissue specificity would be expected for a TCR $\alpha$ -specific cDNA, and the transcript size corresponds well to that of mammalian TRC $\alpha$  genes (18).

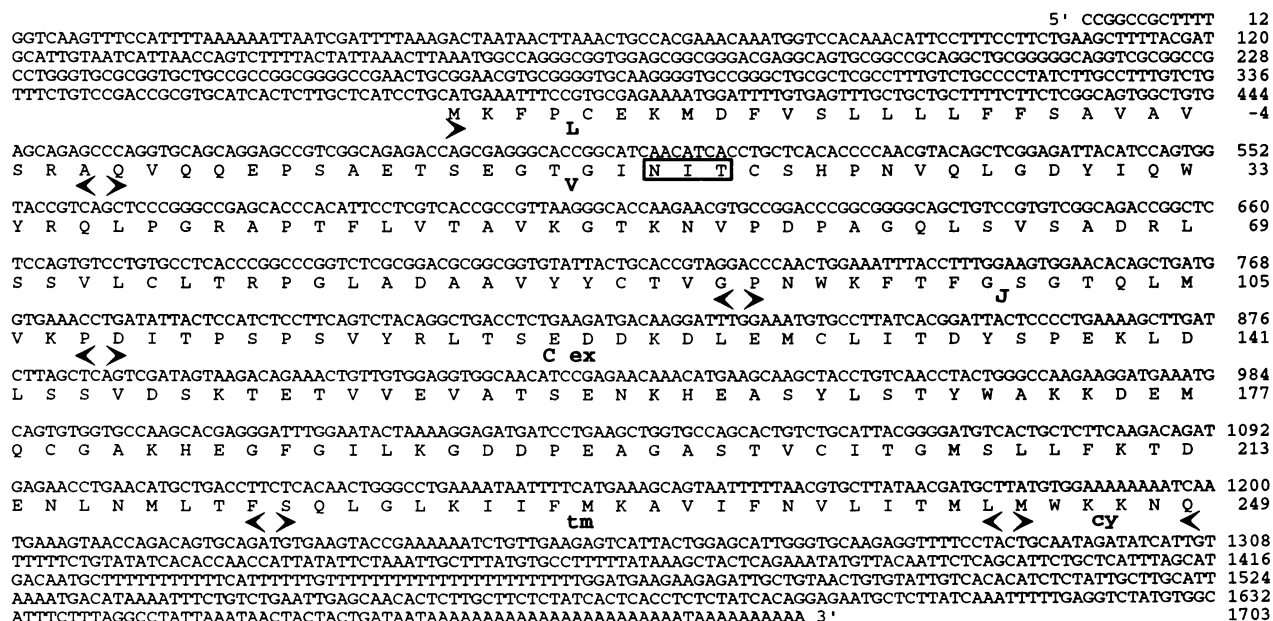


FIG. 2. Nucleotide and predicted amino acid sequence of the 1.7-kb candidate TCR $\alpha$  cDNA. The predicted boundaries between the leader peptide (L), V, and J domains and the extracellular (C ex), transmembrane (tm), and cytoplasmic (cy) segments of the C regions are indicated below the sequence. A potential amino-glycosylation site is boxed, and two possible poly(A) signals are underlined.

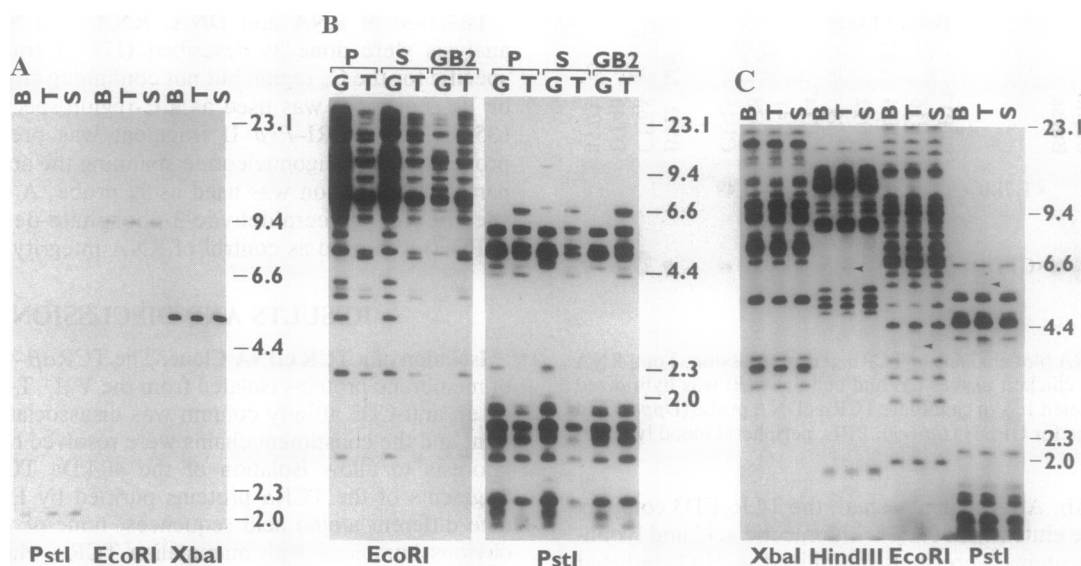


FIG. 3. Southern blot analysis of chicken DNA with TCR $\alpha$  probes. DNA of bursa (B), thymus (T), spleen (S) (A and C) or erythrocytes and T-cell lines (B) was digested with the restriction enzymes indicated and analyzed by Southern blot with radiolabeled probes specific for C $\alpha$  (A) or V $\alpha$  (B and C). Erythrocyte DNA as a germ-line control (G) was compared to a T-cell line (T) DNA of the same chicken strain P, S, or GB2 (B). Arrowheads in C mark faint rearrangement bands. Positions of size markers, as detected by ethidium bromide staining, are indicated in kb.

**Sequence Analysis of the Chicken TCR $\alpha$  cDNA Clone.** Sequence analysis of the 1.7-kb cDNA clone revealed a 375-bp 5' untranslated region, a 503-bp 3' untranslated region, and an open reading frame of 275 amino acids encoded by the 1703-bp-long cDNA (Fig. 2). The predicted molecular mass and isoelectric point are 29.7 kDa and 5.0, respectively. The predicted protein has one potential amino-glycosylation site. These characteristics correlate well with biochemical data indicating that chicken TCR $\alpha$  are acidic molecules of 40 kDa containing amino-linked sugars (5–7).

Comparison of the translated chicken TCR $\alpha$  protein sequence with mammalian TCR $\alpha$  sequences reveals low overall homology, but many amino acid residues important for the structural integrity of TCR $\alpha$  are conserved in the sequence (Fig. 2; see Fig. 6 for detailed information). Regions corresponding to mammalian leader peptide (L), V, J, and C domains can be identified. Based on the homologous mammalian residues, the avian TCR $\alpha$  sequence can be divided into extracellular, transmembrane, and cytoplasmic domains, and invariant cysteine residues exist at positions typical for mammalian TCR $\alpha$  genes (19).

**The Chicken TCR $\alpha$  Locus Contains Multiple Copies of V $\alpha$  and J $\alpha$  Genes and One C $\alpha$  Gene.** Chicken genomic DNA was analyzed by Southern blots with TCR V $\alpha$ , J $\alpha$ , and C $\alpha$ -specific cDNA probes. The C $\alpha$ -specific probe revealed one band (Fig. 3A), suggesting the presence of a single C $\alpha$  gene in chicken as for mammalian TCR $\alpha$  loci (19). In contrast, hybridization of genomic DNA from either T-cell lines (Fig. 3B) or different tissues (Fig. 3C) with a V $\alpha$  region cDNA

probe revealed 20–25 bands, suggesting that the chicken genome contains a large V $\alpha$  family. Multiple bands ( $\approx 20$ ) were also detected when the same Southern blots were hybridized with an oligonucleotide specific for the J $\alpha$  region, suggesting that the chicken genome contains many J $\alpha$  segments (data not shown). Like its mammalian counterpart, the chicken TCR $\alpha$  locus thus appears to be composed of a single C $\alpha$  gene and multiple copies of V $\alpha$  and J $\alpha$  genes.

V $\alpha$  gene rearrangement was evidenced by differences in band patterns between erythrocyte germ-line controls and T-cell lines (Fig. 3B). In addition to the new rearrangement bands observed in T-cell lines, some of the germ-line bands in the erythrocyte controls were missing in the T-cell lines, suggesting rearrangement on both alleles. When DNA isolated from thymus, spleen, or bursa was examined with the V $\alpha$  probe, a faint non-germ-line band was observed in thymic DNA of some, but not all, restriction digests (Fig. 3C, marked by arrowheads).

**Genomic V $\alpha$  and J $\alpha$  Sequence.** To examine the nature of the chicken TCR $\alpha$  recombination signal sequences, V $\alpha$  and J $\alpha$  probes were used to screen chicken genomic libraries. One V $\alpha$ <sup>+</sup> and one J $\alpha$ <sup>+</sup> segment of the EcoRI–Pst I digests from positive clones were subcloned and sequenced (Fig. 4). Both segments were flanked by heptamer and nonamer recombination signal sequences. The length of the spacer region between heptamer and nonamer was 23 bp for V $\alpha$  and 12 bp for J $\alpha$ , typical for the TCR V and J elements. In contrast to mammals, however, a single exon encoded the avian V $\alpha$  segment.

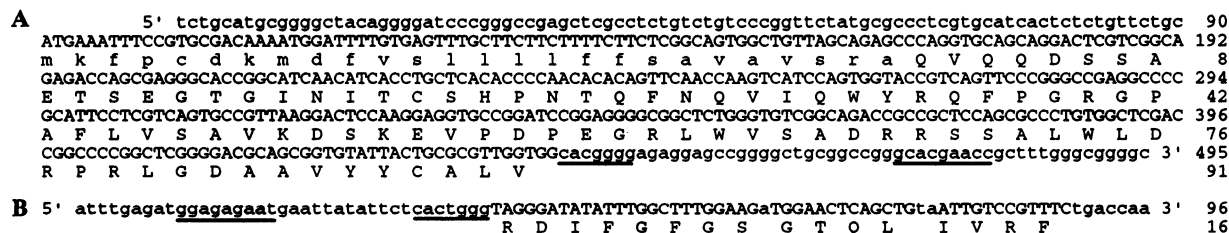
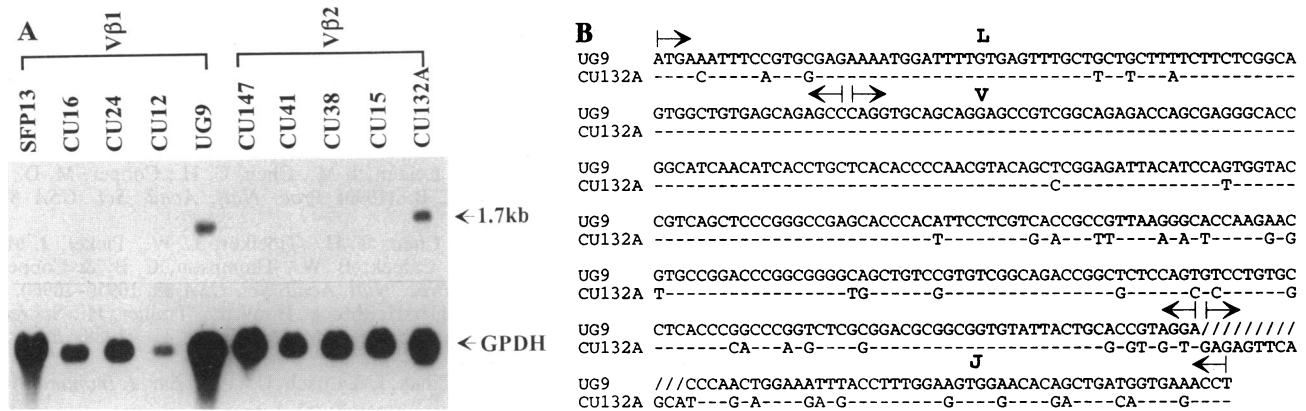


FIG. 4. Nucleotide and amino acid sequences of genomic V $\alpha$  and J $\alpha$  gene segments. The heptamer and nonamer recombination signal sequences are underlined. (A) Sequence of genomic V $\alpha$ 1. V sequence is preceded by leader sequence (lowercase letters). Note that there is no intron sequence between leader and V $\alpha$ . (B) Genomic sequence of J $\alpha$ . Three additional nucleotides (in lowercase letters a and ta) that would change the reading frame appear in the sequence.



**V $\beta$ 1<sup>+</sup> and V $\beta$ 2<sup>+</sup> Cell Lines Can Use V $\alpha$  Segments of the Same Family.** The V $\beta$ 1<sup>+</sup> and V $\beta$ 2<sup>+</sup> subpopulations of  $\alpha\beta$  T cells appear to have different functional potential (1, 9). To analyze the use of V $\alpha$  segments by V $\beta$ 1<sup>+</sup> and V $\beta$ 2<sup>+</sup> cell lines, RNA from a panel of T-cell lines was hybridized with the V $\alpha$  probe isolated from the V $\beta$ 1<sup>+</sup> cell-line UG9 library.

Only two of the cell lines tested reacted with this cDNA probe (Fig. 5A), indicating that the chicken genome contains different  $V_\alpha$  families. A 1.7-kb signal specific for TCR $\alpha$  was detected in the  $V_\beta 1^+$  UG9 cell line and  $V_\beta 2^+$  CU132A cell line. When a TCR $\alpha$ -specific cDNA was isolated from the  $V_\beta 2^+$  CU132A cell line library and its sequence was compared with the TCR $\alpha$  sequence from the  $V_\beta 1^+$  UG9 cell line, homology of 80% at the amino acid level and 89% at the nucleotide level was observed for the predicted  $V_\alpha$  regions (Fig. 5B), indicating that both cell lines used members of the same  $V_\alpha$  family. The level of homology was lower for the  $J_\alpha$  regions of the  $V_\beta 1^+$  and  $V_\beta 2^+$  T-cell lines, being 53% and 75% at the amino acid and nucleotide levels, respectively.

**Evaluation of the Evolutionary Conservation of TCR $\alpha$  Genes.** Comparison of the chicken TCR $\alpha$  sequences with mammalian counterparts revealed remarkably low overall homology, 25–28% (depending on the species compared) at the protein level and 37–41% at the nucleotide level. Although signal peptide

sequences were readily detected in the avian TCR $\alpha$  cDNAs, they did not share significant homology with those of mammals. Also, as noted above, the entire leader and V $\alpha$  sequences were encoded by a single exon. Due to the presence of two methionines in the avian signal sequence, the signal peptide could be either 26 or 19 amino acids long.

The chicken  $V_{\alpha}$  genes were most homologous with members of the human  $V_{\alpha}16$  (42% amino acid homology) and mouse  $V_{\alpha}12$  (43% amino acid homology) families (20). In contrast, the chicken  $V_{\alpha}$  sequences shared only 30% amino acid identity with the chicken immunoglobulin  $\lambda$  chain (21). In human and mouse, 13 amino acids are conserved in >75% of all known  $V_{\alpha}$  gene sequences (22, 23). They are believed to stabilize the structure of the  $V_{\alpha}$  and  $V_{\beta}$  chains and to facilitate their interaction. All of these relatively invariant sequences were found in two of the chicken  $V_{\alpha}$  sequences (Fig. 6A, marked by asterisks), whereas the third  $V_{\alpha}$  sequence had one exchange. Differences between the three chicken  $V_{\alpha}$  sequences were found mainly in the predicted CDR2 and CDR3 regions, whereas their putative FR1 and FR2 regions were almost identical.

On comparison of mammalian and chicken J $\alpha$  sequences, the main diversity was found in the amino-terminal portion that forms the CDR3 region and is susceptible to exonuclease

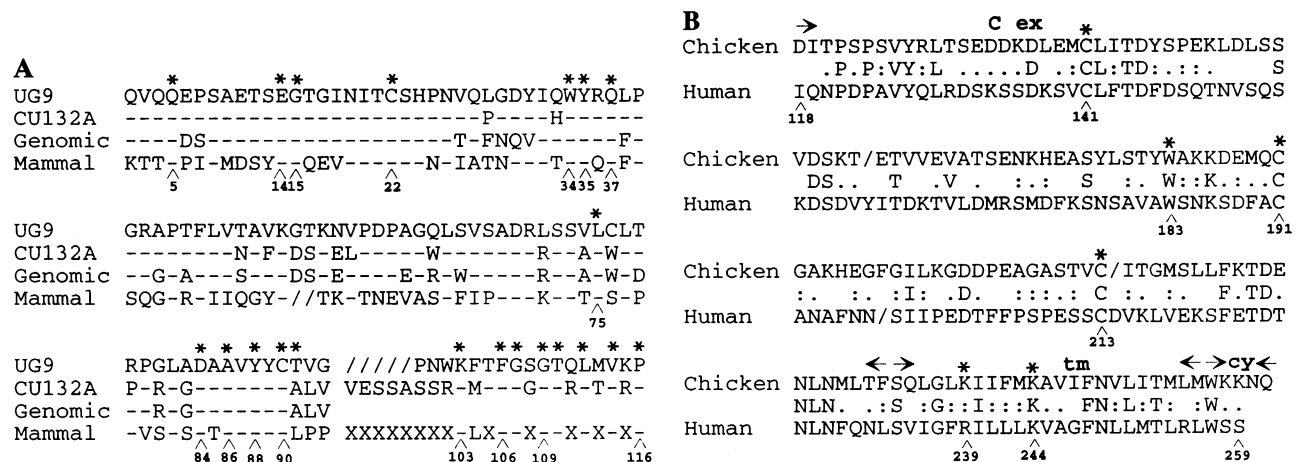


FIG. 6. Amino acid comparison of the predicted chicken TCR $\alpha$  gene products and their corresponding human TCR $\alpha$  sequences. (A) Chicken V $\alpha$  and J $\alpha$  sequences from a V $\rho$ 1+ cell line (UG9), a V $\rho$ 2+ cell line (CU132A), and germ-line sequences were compared with the human V $\alpha$ 16 sequence and the J $\alpha$  50% consensus motif (23), respectively. Conserved amino acids are marked by asterisks and numbered according to Kabat *et al.* (24). Dashes (-) indicate identity, and spacers (/) mark gaps. (B) The chicken C $\alpha$  region is compared with the human C $\alpha$  sequence. Boundaries of extracytoplasmic (C ex), transmembrane (tm), and cytoplasmic (cy) regions are indicated above sequence. A positive relationship (:), a 0 value (.), and a negative relationship (blank), as well as identity between compared residues, are indicated.

degradation. Eight of nine invariant or semi-invariant amino acid residues in mammalian  $J_\alpha$  sequences (23) were conserved in all chicken  $J_\alpha$  regions (Fig. 6A, marked by asterisks). According to the proposed nomenclature for human  $J_\alpha$  sequences (25), the chicken  $J_\alpha$  sequences were most homologous to the human  $J_{\alpha 6}$  and  $J_{\alpha 16}$  segments. The sequence isolated from the  $V_{\beta 2}^+$  T-cell line was 4 amino acids longer than the corresponding sequence of the  $V_{\beta 1}^+$  T-cell line. A similar discrepancy in the length of the CDR3 region was demonstrated for the  $V_{\beta 2}$  segments and could reflect the ontogenetic appearance of these subpopulations because the  $V_{\beta 1}$  and  $V_{\beta 2}$  genes are rearranged sequentially (3).

The chicken  $C_\alpha$  region shared only 25–28% amino acid homology and 37–41% nucleotide homology with the corresponding mammalian sequences. Important residues like the three cysteine residues forming intrachain and interchain disulfide bonds have been conserved in the chicken  $C_\alpha$  sequence (Fig. 6B). Other invariant amino acids included a tryptophan, as well as two positively charged lysines, found in the transmembrane portion of the  $C_\alpha$  region. These charged amino acids are characteristic of TCR $\alpha$  C regions and are required for the association of the TCR chains with the CD3 complex (26).

### CONCLUDING REMARKS

This report describes the cloning of avian TCR $\alpha$  genes in the chicken, thus providing an opportunity to compare the evolution of TCR $\alpha$  genes by examining this locus and its products in a nonmammalian representative. The sequence of the chicken TCR $\alpha$ , although low in overall homology with its mammalian counterparts, shares many conserved structural features with the mammalian TCR $\alpha$  gene that are important for the integrity of the molecule (19). The chicken TCR $\alpha$  gene was found to be rearranged and expressed only in T cells, as is true for mammalian TCR genes. One large  $V_\alpha$  family was identified, members of which are used by both  $V_{\beta 1}^+$  and  $V_{\beta 2}^+$  T-cell lines. The TCR $\alpha$  repertoire is further diversified by the presence of multiple  $J_\alpha$  segments, as in mammals (27). The TCR $\alpha$  genes thus appear to have a much greater potential for combinatorial diversification than the TCR $\beta$  genes in chickens, due to the large number of  $V_\alpha$  and  $J_\alpha$  gene segments.

The high frequency of avian  $\gamma\delta$  T cells (28) and the experimental accessibility to the embryo throughout development make birds a valuable model in which to study the early divergence of the  $\alpha\beta$  and  $\gamma\delta$  T-cell lineages. Genomic analysis of the TCR $\alpha$  locus may reveal elements, such as the  $\alpha$  silencer,  $\alpha$  enhancer, the rearranging element shown to site-specifically recombine with  $\psi J_\alpha$  to delete the  $\delta$  locus  $\delta$  REC,  $\psi J_\alpha$ , T early A, and conserved sequence block (29–31), which may play an important role in the development of  $\alpha\beta$  versus  $\gamma\delta$  T cells. Given the striking similarities of mammalian and chicken TCR genes, the chicken TCR $\delta$  genes should be identifiable by their location between the  $V_\alpha$  and  $J_\alpha$  segments.

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